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2012 Nanotechnology 23 105601
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Facile one-pot preparation, surface functionalization, and toxicity assay of APTS-coated iron oxide nanoparticles

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Received 25 October 2011, in final form 23 December 2011
Published 21 February 2012
Online at stacks.iop.org/Nano/23/105601

Abstract

We report a facile approach to synthesizing 3-aminopropyltrimethoxysilane (APTS)-coated magnetic iron oxide (Fe₃O₄@APTS) nanoparticles (NPs) with tunable surface functional groups for potential biomedical applications. The Fe₃O₄ NPs with a mean diameter of 6.5 nm were synthesized by a hydrothermal route in the presence of APTS. The formed amine-surfaced Fe₃O₄@APTS NPs were further chemically modified with acetic anhydride and succinic anhydride to generate neutral (Fe₃O₄@APTS·Ac) and negatively charged (Fe₃O₄@APTS·SAH) NPs. These differently functionalized NPs were extensively characterized by x-ray diffraction, transmission electron microscopy, Fourier transform infrared spectroscopy, thermogravimetry analysis, zeta potential measurements, and T₂ relaxometry. The cytotoxicity of the particles was evaluated by in vitro 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric viability assay of cells along with microscopic observation of cell morphology. The hemocompatibility of the particles was assessed by in vitro hemolysis assay. We show that the hydrothermal approach enables an efficient modification of APTS onto the Fe₃O₄ NP surfaces and the formed NPs with different surface charge polarities are water-dispersible and colloidally stable. The acetylated Fe₃O₄@APTS·Ac NPs displayed good biocompatibility and hemocompatibility in the concentration range of 0–100 µg ml⁻¹, while the pristine Fe₃O₄@APTS and Fe₃O₄@APTS·SAH particles started to display slight cytotoxicity at a concentration of 10 µg ml⁻¹. The findings from this study suggest that the Fe₃O₄@APTS NPs synthesized by the one-pot hydrothermal route can be surface modified for various potential biomedical applications.

(Some figures may appear in colour only in the online journal)

1. Introduction

Magnetic iron oxide (Fe₃O₄) nanoparticles (IONPs) have been extensively investigated for various applications in biomedical sciences including but not limited to detoxification of biological fluids [1, 2], anti-cancer drug delivery [3–5], hyperthermia [6, 7], and magnetic resonance (MR) imaging [8–12]. For a particular biomedical application, it
is crucial to have an ability to synthesize IONPs with controllable sizes and surface functionalities, since the \textit{in vivo} biomedical performance, especially the blood half-life, opsonization, pharmacokinetics, and biodistribution of the IONPs are closely associated with the particle size and surface modification [1]. A range of different approaches have been employed to synthesize IONPs with controllable size ranging from a few to hundreds of nanometers. These synthetic routes include controlled coprecipitation of Fe(II) and Fe(III) ions at an elevated temperature [13], a successive reduction-oxidation process in a reverse micelle system [14], a thermal decomposition route [15–17], and a hydrothermal method under a higher pressure [18]. These synthetic approaches can be mainly divided into two categories: aqueous phase synthesis and organic phase synthesis. In both cases, the IONPs synthesized either in aqueous solution or in the oil phase have to be surface modified to generate the desired surface functionalities and aqueous dispersity before they can be applied for biomedical applications. Therefore, synthesis of various surface-functionalized IONPs with desired surface functionalities still remains a great challenge.

In order to make IONPs with good water dispersity, which is essential for their biomedical applications, surfactant molecules have been added into the reaction mixture, thereby generating IONPs with desired water dispersity [19]. In most cases, preformed IONPs have been post-functionalized by surfactant molecules [20], silane agent [21, 22] or other small molecular ligands [23–25] to make the IONPs bear reactive functional groups, enabling further bioconjugation chemistry for biomedical applications. There are only a few studies related to the \textit{in situ} functionalization of IONPs with reactive functional ligands by addition of the agents into the reaction mixture for formation of the IONPs [8, 26–28]. For instance, polyethylene glycol (PEG) derivatives have been added into the reaction mixture, which enables the formation of IONPs with carboxyl reactive surface groups [8, 26]. Xie \textit{et al.} added another small molecular ligand 4-methylcatechol into the reaction mixture for the decomposition formation of IONPs to generate functionalized IONPs that enabled conjugation with peptide for MR imaging applications [27]. Pramanik and coworkers developed an approach to forming 3-aminopropyltriethoxysilane (NH$_2$(CH$_2$)$_3$–Si–(OCH$_3$)$_3$, APTS)-coated IONPs by adding APTS into the mixture solution of Fe(II) and Fe(III) ions for controlled coprecipitation formation of IONPs [28]. In general, the post-modification of preformed IONPs is time-consuming and requires additional reactions or steps to process the particles. For the reported one-pot approaches to synthesizing IONPs with reactive surface functional groups, the investigations are mainly limited to high-temperature decomposition formation and controlled coprecipitation formation of IONPs.

In our previous work, we have demonstrated a facile hydrothermal approach to synthesizing Fe$_3$O$_4$ IONPs with relatively uniform size and size distribution under an elevated temperature and pressure [18]. The size of the formed IONPs with high crystallinity can be readily tuned from 15 to 31 nm through variation of the reaction conditions. For biomedical applications, the formed IONPs should be surface functionalized. It is reasonable to deduce that a one-pot approach to synthesizing IONPs with reactive surface functional groups may be realized by addition of a small molecular ligand into the reaction mixture for the hydrothermal synthesis of the IONPs. It is well known that Fe$_3$O$_4$ NPs synthesized by controlled coprecipitation of Fe(II) and Fe(III) ions can be further silanized by APTS by taking advantage of the rich –OH groups on the Fe$_3$O$_4$ particle surfaces [21, 22]; therefore it is hypothesized that by addition of the APTS molecules inside the hydrothermal reaction mixture for the synthesis of IONPs, Fe$_3$O$_4$ NPs with surface amine groups can be easily synthesized.

In this study, we present a simple one-step APTS-assisted hydrothermal approach to synthesizing APTS-coated Fe$_3$O$_4$ NPs (Fe$_3$O$_4$@APTS) with reactive surface amine groups. The APTS modification endowed the Fe$_3$O$_4$ NPs with an excellent water dispersibility and colloidal stability. The surface-reactive amine groups of Fe$_3$O$_4$ NPs were further acetylated and carboxylated by reacting with acetic anhydride and succinic anhydride respectively to generate neutralized and negatively charged particles (scheme 1), which may be used for different biomedical applications. The as-synthesized products were characterized by x-ray diffraction (XRD), transmission electron microscopy (TEM), Fourier transform infrared spectrometry (FTIR), thermogravimetry analysis (TGA), and zeta potential measurements. The $T_2$ relaxometry of the Fe$_3$O$_4$@APTS NPs was measured using a standard multiple spin-echo pulse sequence. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay along with cell morphology observation was used to assess the cytotoxicity of the differently functionalized IONPs, while hemolysis assay was carried out to evaluate the hemocompatibility of the particles. To the best of our knowledge, this is the first report related to the facile one-step synthesis of APTS-coated IONPs with surface-reactive

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{scheme1.png}
\caption{Schematic illustration of the hydrothermal synthesis of Fe$_3$O$_4$@APTS NPs and the subsequent functionalization of the Fe$_3$O$_4$@APTS NPs to form Fe$_3$O$_4$@APTS-Ac and Fe$_3$O$_4$@APTS-SA NPs.}
\end{figure}
amines and the systemic acylation modification of aminated IONPs with different surface charge polarities, which may be used for various biomedical applications.

2. Experimental section

2.1. Materials

All chemicals were analytical grade and used without further purification from commercial sources. Ferrous chloride tetrahydrate \( (\text{FeCl}_2 \cdot 4\text{H}_2\text{O} > 99\%) \), ammonia (28–30\% NH\(_3\) in water solution), triethylamine, acetic anhydride, and dimethyl sulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co., Ltd. APTS and succinic anhydride were obtained from Akros Organics. The KB cells (a human epithelial carcinoma cell line) were from the Institute of Biochemistry and Cell Biology, the Chinese Academy of Science (Shanghai, China). RPMI-1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). MTT was acquired from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. The water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with a resistivity higher than 18.2 M\(\Omega\) cm.

2.2. Synthesis of APTS-coated Fe\(_3\)O\(_4\) NPs

Fe\(_3\)O\(_4\)@APTS NPs were synthesized using a hydrothermal approach described in our previous work with slight modification \[18\]. Typically, \( \text{FeCl}_2 \cdot 4\text{H}_2\text{O} \) (1.25 g) was dissolved in 7.75 ml water. Under vigorous stirring, ammonium hydroxide (6.25 ml) was added, and the suspension was continuously stirred in air for 10 min, allowing the Fe(II) to be oxidized. Then, 2.5 ml of APTS suspension was continuously stirred in air for 10 min, ammonium hydroxide (6.25 ml) was added, and the suspension was continuously stirred in air for 10 min, allowing the Fe(II) to be oxidized. Then, 2.5 ml of APTS was added and the reaction mixture was autoclaved (KH-50 Chemical Reagent Co., Ltd). APTS and succinic anhydride were obtained from Akros Organics. The water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with a resistivity higher than 18.2 M\(\Omega\) cm.

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2.3. Acetylation and carboxylation of Fe\(_3\)O\(_4\)@APTS NPs

The amine groups on the surface of the Fe\(_3\)O\(_4\)@APTS NPs were further acetylated and carboxylated by reacting with acetic anhydride and succinic anhydride respectively according to the protocols described in our previous work \[29\]. Typically, 1 ml of triethylamine was added to the Fe\(_3\)O\(_4\)@APTS (6 mg) solution dispersed in ethanol (5 ml), and the solution was thoroughly mixed. A DMSO solution (5 ml) containing acetic anhydride (1 ml) was dropwise added into the solution of Fe\(_3\)O\(_4\)@APTS mixed with triethylamine while it was being stirred vigorously. The mixture was allowed to react for 24 h. The DMSO, excess reactants, and byproduct were removed from the mixture by five centrifugation/washing/dispersion steps to obtain the Fe\(_3\)O\(_4\)@APTS-Ac NPs finally dispersed in methanol. For carboxylation of the surface amines of the Fe\(_3\)O\(_4\)@APTS NPs, Fe\(_3\)O\(_4\)@APTS NPs (6 mg) dispersed in ethanol (5 ml) was mixed with succinic anhydride (800 mg) dissolved in DMSO (5 ml) under vigorous stirring. The mixture was allowed to react for 24 h. The DMSO and the excess reactants and byproduct were removed from the mixture by five centrifugation/washing/dispersion steps to obtain the Fe\(_3\)O\(_4\)@APTS-SAH NPs that finally dispersed in methanol.

2.4. Characterization techniques

The crystalline structure and the size of the products were determined using XRD by step scan in a D/max 2550 PC x-ray diffractometer (Japan, Rigaku Corp.) with Cu K\(\alpha\) radiation (\(\lambda = 0.1541\) nm) at 40 kV and 200 mA. The scan range (20) was from 25 to 70\(^\circ\)C. The morphology of the formed Fe\(_3\)O\(_4\) NPs was confirmed by TEM imaging using a JEOIL 2010F analytical electron microscope operating at 200 kV. A TEM sample was prepared by placing one drop of diluted Fe\(_3\)O\(_4\) NP suspension (5 \(\mu\)l) onto a 200 mesh carbon-coated copper grid and air-dried before measurement. The Feret diameter of the NPs was measured using the image analysis software ImageJ 1.40G (http://rsb.info.nih.gov/ij/download.html). At least 200 randomly selected NPs in different TEM images were analyzed for each sample to acquire the size distribution histogram. FTIR spectra were recorded using a Nicolet 5700 FTIR spectrometer (Thermo Nicolet Corporation, US) at the wavenumber range of 4000–400 cm\(^{-1}\) at ambient conditions. TGA was carried out using a TG 209 F1 (NETZSCH Instruments Co., Ltd, Germany) thermogravimetric analyzer with a heating rate of 20\(^\circ\)C min\(^{-1}\) and temperature range of 30–900\(^\circ\)C under nitrogen gas. Zeta potential measurements and dynamic light scattering were performed using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, UK) equipped with a standard 633 nm laser. \(\tau_2\) relaxation time was performed using an NMI20-Analyt NMR Analyzing & Imaging system (Shanghai Niumag Corporation). The instrumental parameters were set as follows: a 0.5 T magnet, point resolution = 156 mm \(\times\) 156 mm, section thickness = 0.6 mm, \(TE = 60\) ms, \(TR = 4000\) ms, number of acquisitions = 1. The \(\tau_2\) relaxation time was calculated from the linear slope of the inverse \(T_2\) (1/\(T_2\)) relaxation time versus the Fe molar concentration. The Fe concentration of the Fe\(_3\)O\(_4\)@APTS NPs was analyzed using a PRODIGY inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Teledyne Leeman Labs, USA) after aqua regia treatment. The stability of the functionalized Fe\(_3\)O\(_4\) NPs was assessed by dispersing them in water, phosphate buffered saline (PBS), and FBS solution for a period of time of up to one month.

2.5. Cytotoxicity assay

The KB cells were continuously grown in a 50 ml culture flask in regular RPMI-1640 medium supplemented with 10%
heat-inactivated FBS, 100 U ml\(^{-1}\) penicillin, and 100 U ml\(^{-1}\) streptomycin. An MTT assay was used to quantify the viability of the cells upon treatment with the Fe\(_3\)O\(_4\) NPs with different surface functional groups. Briefly, \(1 \times 10^4\) KB cells per well were seeded into a 96-well plate. After overnight incubation to bring the cell to 80% confluence, the medium was replaced with fresh medium containing differently functionalized Fe\(_3\)O\(_4\) NPs with different concentrations (1, 10, 50 and 100 µg ml\(^{-1}\)). After 24 h incubation at 37°C, the metabolically active cells were then detected by adding MTT to each well. The assays were carried out according to the manufacturer’s instructions and the absorbance of each well was measured using a Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, USA) at 570 nm. The mean and standard deviation for the triplicate wells were reported. One-way analysis of variance (ANOVA) statistical analysis was performed to detect the difference between the differently functionalized Fe\(_3\)O\(_4\) NPs and the control without treatment. A \(p\) value of 0.05 was considered as a statistically significant level.

After treatment with the functionalized Fe\(_3\)O\(_4\) NPs for 24 h, the cell morphology was observed by phase contrast microscopy (Leica DM IL LED inverted phase contrast microscope). The magnification was set at 200× for all samples.

2.6. Hemolysis assay

Fresh human blood stabilized with heparin was kindly provided by Shanghai First People’s Hospital (Shanghai, China). A pre-treatment was conducted to obtain healthy red blood cells (HRBCs) for hemolysis assay according to the literature [30]. Briefly, HRBCs were isolated from fresh human blood by centrifugation at 2000 rpm for 10 min and purified by successively rinsing with PBS five times. After that, the HRBCs were diluted ten times with PBS buffer. The diluted HRBC suspension (0.1 ml) was added to 1.5 ml of water (positive control), pure PBS (negative control), and PBS buffer containing Fe\(_3\)O\(_4\)@APTS, Fe\(_3\)O\(_4\)@APTS-Ac, and Fe\(_3\)O\(_4\)@APTS-SAH NPs, respectively with a concentration ranging from 50 to 400 µg ml\(^{-1}\). After a gentle shaking, the mixtures were kept still for 2 h at room temperature. Then, after centrifugation of the mixture (1000 rpm, 1 min), the absorbance of the supernatant (hemoglobin) was recorded by a Perkin Elmer Lambda 25 UV–vis spectrometer. The hemolysis percentages of the samples were calculated by dividing the difference in absorbances between the sample and the negative control by the difference in absorbances at 541 nm between the positive and negative controls.

3. Results and discussion

3.1. Synthesis and characterization of Fe\(_3\)O\(_4\)@APTS NPs with different surface functional groups

The crystallinity of the formed APTS-coated Fe\(_3\)O\(_4\) NPs was characterized using XRD (figure 1). From the XRD pattern, the lattice spacing calculated from the diffraction peaks observed at 30, 35.6, 37.1, 43, 53.5, 57, and 62.7 matched the [220], [311], [222], [400], [422], [511], and [440] planes of Fe\(_3\)O\(_4\) crystals, respectively. The XRD patterns were consistent with those reported in the literature [18, 31–33], which can be assigned to the magnetite phase of iron oxide [12, 18, 34, 35]. Based on the major diffraction

![Figure 1. XRD pattern of Fe\(_3\)O\(_4\)@APTS NPs.](image1)

![Figure 2. TEM micrograph and size distribution histogram of the Fe\(_3\)O\(_4\)@APTS NPs.](image2)
Fe(II) and Fe(III) ions [36] that were then silanized with NPs (diameter \( = \) the –NH$_2$ groups introduced from the APTS. In addition, the bands of the Fe–O bond could also be clearly seen at 594 and 462 cm$^{-1}$ are due to the O–H stretching and bending vibrations of the C–N bond. In contrast, in the FTIR spectrum of the uncoated Fe$_3$O$_4$ NPs, the absorptions at 3433 and 1630 cm$^{-1}$ are due to the O–H stretching and bending vibrations of physically adsorbed H$_2$O and surface –OH groups. The stronger absorption bands at 3433 and 1630 cm$^{-1}$ are the –NH$_2$ groups introduced from the APTS. In addition, the bands of the Fe–O bond could also be clearly seen at 594 and 462 cm$^{-1}$. All of these bands revealed that the surface of the Fe$_3$O$_4$ NPs was successfully modified with APTS, in agreement with the results reported in the literature [28], where the APTS was modified onto preformed Fe$_3$O$_4$ NPs.

Modification of APTS on the surface of Fe$_3$O$_4$ NPs was further confirmed by TGA analysis. In order to demonstrate the efficiency of APTS coating onto the Fe$_3$O$_4$ NPs synthesized by the hydrothermal approach, preformed Fe$_3$O$_4$ NPs (diameter = 8.4 nm) via controlled coprecipitation of Fe(II) and Fe(III) ions [36] that were then silanized with APTS (for short, Fe$_3$O$_4$@APTS$_2$ NPs) and the Fe$_3$O$_4$ NPs formed using the same hydrothermal method in the absence of APTS were included for comparison (figure 4). It can be seen that the weight loss of the Fe$_3$O$_4$@APTS$_2$ NPs synthesized by the one-pot hydrothermal approach is much higher than that of the Fe$_3$O$_4$@APTS$_2$ NPs (6%). This suggests that the former NPs have much more APTS coating than the latter. In contrast, the naked Fe$_3$O$_4$ NPs do not have any significant weight loss at temperatures as high as 800$^\circ$C. The high amount of APTS coating onto the Fe$_3$O$_4$ NPs would result in a more dispersed distribution of –NH$_2$ moieties on the particle surfaces, favoring further functionalization and surface modification of the IONPs for various biomedical applications.

To prove our hypothesis that the densely aminated Fe$_3$O$_4$ NPs can be further functionalized, we then reacted the Fe$_3$O$_4$@APTS surface amines with acetic anhydride and succinic anhydride to generate acetylated Fe$_3$O$_4$@APTS@Ac and carboxylated Fe$_3$O$_4$@APTS@SAH NPs, respectively [29]. Zeta potential measurements were employed to confirm the surface charge polarity changes after reaction (table 1). It is clear that Fe$_3$O$_4$@APTS NPs are positively charged with a mean surface potential of 26.7 mV due to an abundance of APTS amine groups anchored on the NP surfaces. After acetylation of the APTS amines, the formed Fe$_3$O$_4$@APTS@Ac NPs were fairly well neutralized with a slightly negative surface potential (–3.2 mV). The highly positive surface potential of the Fe$_3$O$_4$@APTS NPs was switched to highly negative (–25.6 mV) when the APTS amines were reacted with succinic anhydride to

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fe$_3$O$_4$@APTS</th>
<th>Fe$_3$O$_4$@APTS-Ac</th>
<th>Fe$_3$O$_4$@APTS-SAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta potential (mV)</td>
<td>26.7 ± 1.4</td>
<td>–3.2 ± 1.5</td>
<td>–25.6 ± 0.6</td>
</tr>
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Figure 3. FTIR spectra of the Fe$_3$O$_4$ NPs synthesized in the absence of APTS (a) and the Fe$_3$O$_4$@APTS NPs (b).

Figure 4. TGA curves of the Fe$_3$O$_4$@APTS NPs synthesized by the one-pot hydrothermal approach, the Fe$_3$O$_4$@APTS$_2$ NPs formed by APTS silanization of preformed Fe$_3$O$_4$ NPs via controlled coprecipitation of Fe(II) and Fe(III) ions, and the naked Fe$_3$O$_4$ NPs synthesized by the hydrothermal approach in the absence of APTS.
generate carboxyl-functionalized Fe$_3$O$_4$@APTS-SA H NPs. This suggests that via post-modification of the APTS amines on the surface of Fe$_3$O$_4$@APTS NPs, the surface charge polarity can be modulated to be neutral or negative. The Fe$_3$O$_4$@APTS NPs with amine, acetyl, and carboxyl surface functional groups all displayed a good colloidal stability in water, PBS buffer, and FBS solution after being stored at 4°C for at least one month. The photograph of the Fe$_3$O$_4$@APTS NPs with different surface groups dispersed in different media shows that the particles do not precipitate even after a month’s storage at 4°C (figure 5). Dynamic light scattering was also employed to check the hydrodynamic sizes of the particles from time to time. We showed that the hydrodynamic sizes of the Fe$_3$O$_4$@APTS, Fe$_3$O$_4$@APTS-Ac, and Fe$_3$O$_4$@APTS-SA H NPs, measured to be 247.8 ± 18.6 nm, 337.1 ± 56.5 nm, and 235.6 ± 42.2 nm, respectively, did not have any significant changes over a period of one month. The larger hydrodynamic sizes than those measured by TEM for all the NPs with different surface functional groups are due to the fact that TEM only measures the inorganic Fe$_3$O$_4$ core particles, while dynamic light scattering measures the entire NP cluster size with a thick hydrated shell. Our study clearly demonstrates their good colloidal stability, which is essential for biomedical applications.

3.2. $T_2$ relaxivity of Fe$_3$O$_4$@APTS NPs

The magnetic behavior of Fe$_3$O$_4$ or Fe$_3$O$_4$-based NPs is very important for their biomedical applications, especially in MR imaging. To evaluate the possibility of using APTS-coated Fe$_3$O$_4$ NPs as a potential $T_2$-based contrast agent for MR imaging, the transverse relaxation time ($T_2$) of Fe$_3$O$_4$@APTS was measured at 0.5 T with a spin-echo pulse sequence. The measured $T_2$ data were used to calculate the transverse relaxivity ($r_2$) (the transverse relaxation rate per mM of iron), which represents the efficiency of the NPs as a contrast agent. As shown in figure 6(a), the signal intensity of the $T_2$-weighted MR images dramatically decreases with Fe concentration. The $T_2$ relaxation rate (1/$T_2$) as a function of the Fe concentration in Fe$_3$O$_4$@APTS NPs (figure 6(b)) shows that the relaxation rate increases linearly with the Fe concentration with a slope ($r_2$) of 83.8 mM$^{-1}$ s$^{-1}$, which is slightly lower than that of the Fe$_3$O$_4$ NPs (100.4 mM$^{-1}$ s$^{-1}$) synthesized by controlled coprecipitation of Fe(II) and Fe(III) ions reported in our previous work [10]. The slightly lower $r_2$ may be due to the fact that the APTS coating onto the surfaces of the Fe$_3$O$_4$ NPs shields water molecules from accessing their surfaces. Our results suggest that Fe$_3$O$_4$@APTS NPs could be used as a $T_2$-shortening agent because of their small size and relatively large $r_2$ value when compared with other Fe$_3$O$_4$ NPs coated with polymer multilayers [10].

3.3. Cytotoxicity of Fe$_3$O$_4$@APTS NPs with different surface groups

The amine moieties anchored onto the NPs could yield cytotoxicity and possible non-specific membrane binding, limiting their biological applications. In our previous studies,
we have shown that decreasing the surface charge of amine-terminated poly(amidoamine) dendrimers toward neutral can reduce their toxicity [29, 37]. Similarly, in this study the APTS amine groups on the \( \text{Fe}_3\text{O}_4 @\text{APTS} \) NP surfaces were transformed to acetamide and carboxyl groups, which may result in improved biocompatibility of the particles.

MTT assay was used to assess the viability of KB cells treated with \( \text{Fe}_3\text{O}_4 @\text{APTS} \), \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{Ac} \), and \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{SAH} \) NPs, respectively (figure 7). PBS with similar volume to that used to disperse the particles was used as a control for statistical comparison with each cell sample treated with differently functionalized \( \text{Fe}_3\text{O}_4 \) NPs with different concentrations. Compared to the control, there was no statistically significant difference in the viability of cells treated by \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{Ac} \) NPs at the studied concentration ranging from 0 to 100 \( \mu\text{g ml}^{-1} \) (\( p > 0.05 \)), while the pristine \( \text{Fe}_3\text{O}_4 @\text{APTS} \) and \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{SAH} \) NPs started to display slight cytotoxicity at a concentration of 10 \( \mu\text{g ml}^{-1} \) (\( p < 0.05 \)), suggesting that neither amine nor carboxyl functionalization of the APTS-coated \( \text{Fe}_3\text{O}_4 \) NPs is favorable for cell proliferation at high concentrations. In general, the cytotoxicity of NPs with amine groups on their surface stems from the strong electrostatic interaction between the positively charged NPs and the negatively charged cell membranes [37, 38]. Therefore, it is reasonable to see that the aminated \( \text{Fe}_3\text{O}_4 @\text{APTS} \) NPs have a slight cytotoxicity at higher concentrations. However, the reason for the slight cytotoxicity of the carboxylated \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{SAH} \) NPs at higher concentration is still unclear. Overall, our study suggests that the acetylated \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{Ac} \) NPs with neutral surface charge have good biocompatibility.

The cytotoxicity of the APTS-coated \( \text{Fe}_3\text{O}_4 \) NPs with different surface functionalizations was further confirmed by observation of the morphology of cells treated with these particles. Figure 8 shows phase contrast microscopic images of KB cells treated with \( \text{Fe}_3\text{O}_4 @\text{APTS} \), \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{Ac} \), and \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{SAH} \) NPs, respectively (figure 7). PBS with similar volume to that used to disperse the particles was used as a control for statistical comparison with each cell sample treated with differently functionalized \( \text{Fe}_3\text{O}_4 \) NPs with different concentrations. Compared to the control, there was no statistically significant difference in the viability of cells treated by \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{Ac} \) NPs at the studied concentration ranging from 0 to 100 \( \mu\text{g ml}^{-1} \) (\( p > 0.05 \)), while the pristine \( \text{Fe}_3\text{O}_4 @\text{APTS} \) and \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{SAH} \) NPs started to display slight cytotoxicity at a concentration of 10 \( \mu\text{g ml}^{-1} \) (\( p < 0.05 \)), suggesting that neither amine nor carboxyl functionalization of the APTS-coated \( \text{Fe}_3\text{O}_4 \) NPs is favorable for cell proliferation at high concentrations. In general, the cytotoxicity of NPs with amine groups on their surface stems from the strong electrostatic interaction between the positively charged NPs and the negatively charged cell membranes [37, 38]. Therefore, it is reasonable to see that the aminated \( \text{Fe}_3\text{O}_4 @\text{APTS} \) NPs have a slight cytotoxicity at higher concentrations. However, the reason for the slight cytotoxicity of the carboxylated \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{SAH} \) NPs at higher concentration is still unclear. Overall, our study suggests that the acetylated \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{Ac} \) NPs with neutral surface charge have good biocompatibility.

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![Figure 7](image_url) "Figure 7. MTT assay of KB cell viability after treatment with \( \text{Fe}_3\text{O}_4 @\text{APTS} \), \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{Ac} \), and \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{SAH} \) NPs for 24 h. The mean and standard deviation for the triplicate wells are reported. The data are expressed as mean \pm SD. Statistical significance was calculated using the ANOVA method and is indicated by (*) for \( p < 0.05 \)."

![Figure 8](image_url) "Figure 8. Phase contrast microscopic images of KB cells treated with PBS buffer (a), \( \text{Fe}_3\text{O}_4 @\text{APTS} \) NPs (b), \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{Ac} \) NPs (c), and \( \text{Fe}_3\text{O}_4 @\text{APTS}-\text{SAH} \) NPs (d) with a concentration of 10 \( \mu\text{g ml}^{-1} \)."
good biocompatibility of the acetylated Fe$_3$O$_4$@APTS-Ac NPs, in agreement with the above MTT assay data.

3.4. Hemolysis assay

Hemocompatibility is one of the most important issues that has to be addressed before the materials can be used for in vivo applications, especially for materials required to contact blood. Figure 9 shows the hemolytic behaviors of Fe$_3$O$_4$@APTS and the derivatives at different concentrations (50, 100, 200, and 400 µg ml$^{-1}$). It is apparent that there is no visible hemolytic effect for any of the particles at a concentration of 400 µg ml$^{-1}$ (figure 9(d)). The hemolysis effect of the samples was quantitatively evaluated by measuring the absorbance of the supernatant at 541 nm (figures 9(a)–(c)). At a concentration up to 200 µg ml$^{-1}$, the hemolysis percentages of Fe$_3$O$_4$@APTS, Fe$_3$O$_4$@APTS-Ac, and Fe$_3$O$_4$@APTS-SAH NPs were all less than 5%. However, differently from the visible photograph shown in figure 9(d), when the particle concentration was increased to 400 µg ml$^{-1}$, the hemolytic activities of Fe$_3$O$_4$@APTS-SAH and Fe$_3$O$_4$@APTS were determined to be 9.1% and 12.9%, respectively. In contrast, at the same concentration, the Fe$_3$O$_4$@APTS-Ac NPs displayed a very low percentage of hemolysis (1.4%), suggesting their excellent hemocompatibility at higher concentrations. The negligible hemolytic activity of Fe$_3$O$_4$@APTS-Ac NPs in a wide concentration range of 0–400 µg ml$^{-1}$ along with their excellent cytocompatibility may enable them to be used for various biomedical applications.

4. Conclusion

We have synthesized APTS-coated Fe$_3$O$_4$ NPs with a mean diameter of 6.5 nm using a facile one-pot hydrothermal method. The formed APTS-coated Fe$_3$O$_4$ NPs can be further functionalized with acetyl and carboxyl groups after reaction of the surface APTS amines with acetic anhydride and succinic anhydride, respectively. The structure, morphology, composition, and surface properties of the formed particles were characterized by XRD, TEM, FTIR, TGA, and zeta potential measurements. We showed that the presence of APTS molecules not only enables much more efficient APTS coating of the particles with reactive amine groups, but also significantly limits the particle growth to form particles with a much smaller size. Cytotoxicity and hemolytic assay results show that acetylation of the APTS amine groups on the particle surfaces can significantly improve the particles’ cytocompatibility and hemocompatibility. With the high $T_2$ relaxivity of the APTS-coated IONPs, the tunable amine chemistry, and the ability to generate biocompatible NPs, we anticipate that the formed APTS-coated IONPs may be further biofunctionalized for various biomedical applications, especially for MR imaging and diagnosis of diseases.
Acknowledgments

This research is financially supported by the National Natural Science Foundation of China (81101150, 20974019), the Nano Specialized Research Fund of Shanghai Science and Technology Commission (1052nm05800, 11nm0506400), the Fundamental Research Funds for the Central Universities (for MS, RG, XC, and XS), the Shanghai Natural Science Foundation (11ZR1429300), and the Innovation Funds of Donghua University Doctorate Dissertation of Excellence (BC201104 for HC). MS acknowledges the support from Shanghai Bai Yu Lan foundation (2010B003). KL acknowledges the Shanghai Songjiang Medical Climbing Program (2011PD04). XS gratefully acknowledges the FCT and Santander bank for the Chair in Nanotechnology.

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